

REPORTS

Bax and Adenine Nucleotide Translocator Cooperate in the Mitochondrial Control of Apoptosis

P.D. 25-09-1998
P. 2027-2031

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The proapoptotic Bax protein induces cell death by acting on mitochondria. Bax binds to the permeability transition pore complex (PTPC), a composite proteaceous channel that is involved in the regulation of mitochondrial membrane permeability. Immunodepletion of Bax from PTPC or purification of PTPC from Bax-deficient mice yielded a PTPC that could not permeabilize membranes in response to atractyloside, a proapoptotic ligand of the adenine nucleotide translocator (ANT). Bax and ANT coimmunoprecipitated and interacted in the yeast two-hybrid system. Ectopic expression of Bax induced cell death in wild-type but not in ANT-deficient yeast. Recombinant Bax and purified ANT, but neither of them alone, efficiently formed atractyloside-responsive channels in artificial membranes. Hence, the proapoptotic molecule Bax and the constitutive mitochondrial protein ANT cooperate within the PTPC to increase mitochondrial membrane permeability and to trigger cell death.

Pro- and antiapoptotic members of the Bcl-2 family have pleiotropic effects on caspase activation cascades regulated by the apopto-

some, on cellular redox potentials, and on the barrier function of mitochondrial membranes (1-9). The proapoptotic protein Bax redistributes to and acts on mitochondria to induce cell death (6-9). Bax induces all mitochondrial hallmarks of apoptosis when overexpressed in cells, including the dissipation of the mitochondrial inner transmembrane potential ($\Delta\Psi_m$) (1) and the release of cytochrome c through the outer mitochondrial membrane (6-9).

These effects of Bax are the same as those of agents that open the permeability transition (PT) pore, a structure involved in the control of apoptosis (2). Accordingly, recombinant Bax (10) and atractyloside (Atr)—a PT pore-

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opening agent that acts on one of the PTPC components, ANT (also called ATP/ADP carrier)—induced $\Delta\Psi_m$ dissipation and nuclear apoptosis when microinjected into cells with an efficiency of $\geq 80\%$ (Fig. 1A). We found no such effects for a mutant inactive Bax protein (Δ IGDE) (10), which lacks a motif within the BH3 domain essential for homodimerization, nor for a Bax protein ($\Delta\alpha 5/6$) lacking the putative pore-forming domain $\alpha 5/6$ between BH1 and BH2 (10) (Fig. 1A).

The mitochondrial and nuclear effects of Bax are prevented ($<10\%$) by three prototypic inhibitors of the PT pore: cyclosporin A (CsA) (8, 11, 12), the nonimmunosuppressive CsA derivative *N*-methyl-4-Val-CsA (m.CsA, which can still bind to mitochondrial cyclophilin D) (11), and bongkreic acid (BA), which binds to another site of the ANT than Atr (13) (Fig. 1A). These three inhibitors also prevented the effects of Bax on isolated mitochondria: $\Delta\Psi_m$ reduction (Fig. 1, B and C), release of cytochrome c (Fig. 1E), and release of a factor different from cytochrome c (2) that causes isolated nuclei to fragment their DNA in a cell-free system [apoptosis-inducing factor (AIF)] (Fig. 1D). In contrast, the caspase inhibitor z-VAD.fmk did not inhibit the $\Delta\Psi_m$ dissipation induced by Bax microinjection, although it did inhibit nuclear apoptosis. Bax-mediated effects on mitochondria and nuclei also did not require *de novo* protein synthesis (Fig. 1A). Thus, the effect of

Bax on mitochondria involves a close functional interaction with the pharmacological targets of BA and CsA.

The PTPC contains several constitutive mitochondrial membrane proteins, including the inner transmembrane protein ANT and the soluble matrix protein cyclophilin D (11, 12). PTPC isolated from the rat brain normally contains Bax (14) and can be reconstituted into liposomes, where it regulates liposomal membrane permeability similarly to the PT pore in intact mitochondria (14, 15). Addition of the hydrophilic ANT ligand Atr (which binds to the intermembrane face of the ANT) (13) caused liposomes containing PTPC (16) to release small molecules such as [3 H]glucose (180 daltons, Fig. 2A) or DiOC₆(3) (573 daltons, Fig. 2, C and D), but not [3 H]inulin (5200 daltons, Fig. 2B), indicating that PTPC liposomes contain ANT with the Atr-binding site at the surface, which is in the same orientation as in mitoplasts. When Bax was immunodepleted from PTPC before PTPC reconstitution into liposomes, the resulting complex failed to respond to Atr (Fig. 2, A and D). This cannot be due to depletion of the Atr target because Bax-immunodepleted PTPC still contained ANT molecules (Fig. 2C) and because recombinant Bax (but not recombinant Bcl-2) added to Bax-depleted PTPC could restore a CsA- or BA-inhibitable response to Atr (Fig. 2D). Moreover, this is a selective effect, because the Bax-depleted

PTPC continued to respond to the reactive oxygen species donor *tert*-butylhydroperoxide (*t*-BHP) (Fig. 2, A and D), the thiol-cross-linking agent diamide (Fig. 2D), and caspases 1 or 3 (17). Bcl-2 prevented the *t*-BHP-induced membrane permeabilization, even when Bax was depleted from the PTPC (Fig. 2D), in accord with the fact that Bcl-2 can exert at least some of its death-antagonistic effects in the absence of Bax (18, 19).

When purified from the brain of Bax-deficient (*Bax*^{-/-}) mice and reconstituted into liposomes, PTPC did not respond to Atr, whereas PTPC from control mice (*Bax*^{+/+}) responded normally (Fig. 3A). Again, this defect is selective for Atr (but not *t*-BHP or diamide) and could be reverted by the addition of Bax (but not the inactive Bax mutants Δ IGDE or $\Delta\alpha 5/6$; Fig. 3A), thus confirming the data obtained with Bax-immunodepleted rat PTPC. In addition, Atr did not disrupt the $\Delta\Psi_m$ of liver mitochondria purified from *Bax*^{-/-} mice in conditions in which it caused a $\Delta\Psi_m$ collapse in mitochondria from control (*Bax*^{+/+}) mice (Fig. 3B). Further genetic evidence for the Bax-ANT cooperation was obtained in yeast (*Saccharomyces cerevisiae*) cells in which all three genes coding for ANT isoenzymes were inactivated. Transfection of wild-type yeast with galactose-inducible Bax plasmid resulted in markedly reduced colony formation on galactose plates ($<1\%$ survival after 24 hours of Bax overexpression), con-

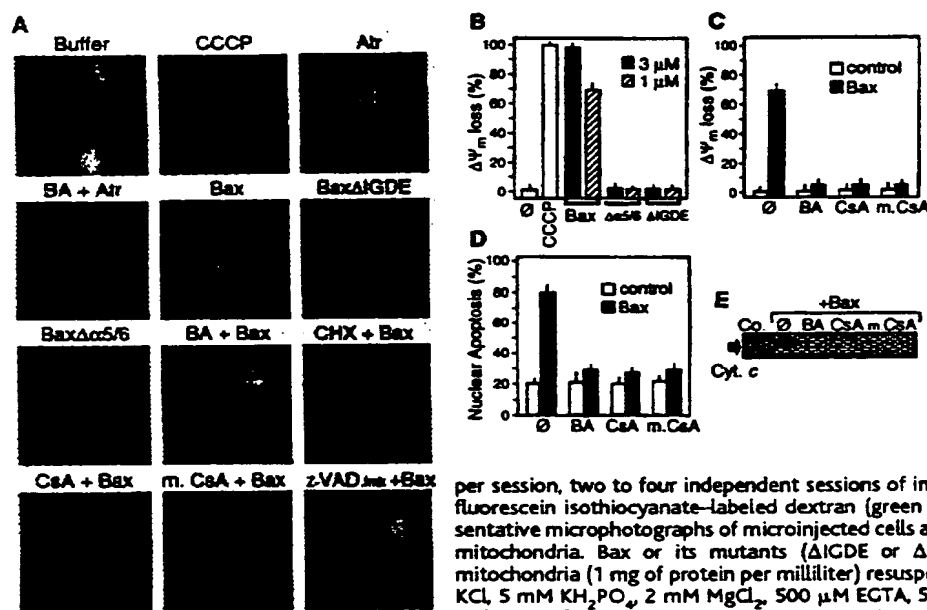


Fig. 1. Bax effects are neutralized by inhibitors of the PT pore. (A) Microinjection of Bax or Atr induces $\Delta\Psi_m$ dissipation and nuclear apoptosis in a BA-inhibitable fashion. Rat-1 fibroblast cells were microinjected with Atr (50 μ M), Bax (1 μ M), mutant Bax proteins (Δ IGDE or $\Delta\alpha 5/6$), or buffer only. BA (50 μ M), CsA (1 μ M), m.CsA (1 μ M), SDZ 220-384; Novartis, Basel, Switzerland), the caspase inhibitor z-VAD.fmk (50 μ M; Bachem, Basel, Switzerland), or the protein synthesis inhibitor cycloheximide (CHX, 35 μ M) were added to the culture medium 30 min before microinjection into the cytoplasm (pressure 150 hPa; 0.2 s). After microinjection, cells were cultured for 90 min and stained for 10 min with the potential-sensitive dye CMXRos [100 nM, red fluorescence; negative control obtained with 50 μ M protonophore (CCCP) added before staining] and the DNA-intercalating Hoechst 33258 dye (1 μ M, blue fluorescence). Microinjected viable cells (100 to 200

per session, two to four independent sessions of injection) were identified by inclusion of 2.5% (w/v) fluorescein isothiocyanate-labeled dextran (green fluorescence) in the injectate (not shown). Representative microphotographs of microinjected cells are shown. (B) Bax causes $\Delta\Psi_m$ reduction in isolated mitochondria. Bax or its mutants (Δ IGDE or $\Delta\alpha 5/6$) were added to purified (32) mouse liver mitochondria (1 mg of protein per milliliter) resuspended in 220 mM sucrose, 68 mM mannitol, 10 mM KCl, 5 mM KH₂PO₄, 2 mM MgCl₂, 500 μ M EGTA, 5 mM succinate, 2 μ M rotenone, 10 mM Hepes (pH 7.2). The $\Delta\Psi_m$ was monitored by measuring the quenching of rhodamine 123 fluorescence (5 μ M) (5). The protonophore CCCP (10 μ M) served as positive control (100%) of $\Delta\Psi_m$ dissipation (mean \pm SD of triplicates; representative of six experiments). 198, negative control. (C) Inhibitory effect of BA (50 μ M), CsA (1 μ M), and m.CsA (1 μ M) on the Bax-induced (1 μ M, 60 min) $\Delta\Psi_m$ dissipation. The $\Delta\Psi_m$ dissipation was measured as in (B). (D) Inhibitory effect of BA, CsA, or m.CsA on the mitochondrial release of apoptosis-inducing factor. The supernatant of mitochondria treated with Bax or PT pore inhibitor, or both as in (C) was added to isolated HeLa nuclei, and DNA loss occurring after 90 min of incubation was assessed by cytofluorometric analysis of propidium iodide-stained nuclei (33). (E) Bax-induced cytochrome c release and its inhibition by BA, CsA, and m.CsA (Co., control). The supernatants of mitochondria incubated as in (D) were subjected to immunoblot analysis (34).

sistent with previous reports that Bax kills yeast cells through a mitochondria-targeting mechanism (9, 20). In contrast, ANT-deficient yeast were completely resistant to Bax, forming colonies comparable to cells transformed with control plasmids (Fig. 3C). These data emphasize the functional interaction between Bax and the Atr target ANT.

In mammalian cells, Bax and ANT coimmunoprecipitated (Figs. 2C and 4A), thus supporting our observation that Bax copurified with PTPC, which contains ANT protein (14) (Fig. 2C). We observed the interaction between ANT and Bax in different tissues including rat brain (Fig. 2C), mouse liver, and human HT-29 colon cancer cells (Fig. 4A). In this latter cell line, ANT and Bcl-2 also coimmunoprecipitated (21) (Fig. 4A). This interaction is likely to be direct, on the basis of two observations. First, in a yeast two-hybrid system (22) a fragment (amino acids 105 to 156) of the ubiquitously expressed human ANT2 isoenzyme interacted with Bax and with its close homolog Bak as well as with Bcl-2 (Fig. 4B). Second, recombinant Bax and purified ANT cooperated to reproduce some features of the PTPC in vitro. For the latter experiments, ANT was purified to >95% homogeneity and found to be uncontaminated by other proteins typically contained in PTPC (cyclophilin D, porin, and Bax) (23). When incorporated into liposomal membranes, only the combination of purified ANT and Bax, but neither molecule alone, efficiently permeabilized the liposomal membranes in the presence of Atr (Fig. 4C). Thus, in these conditions, ANT and Bax are necessary and sufficient to form Atr-sensitive pores in

artificial membranes. It is of note that in this system Bax cannot be replaced by Bcl-2, Bax Δ IGDE, or Bax Δ 5/6 (Fig. 4C), supporting the idea that homodimerization of Bax (deficient in Bax Δ IGDE) and pore

formation (deficient in Bax Δ 5/6) are required for the proapoptotic effect of Bax (6–8, 10).

When incorporated into artificial membranes, relatively large amounts of Bax can

Fig. 3. Genetic evidence for the functional interaction between Bax and ANT. (A) Comparison of purified PTPC from wild-type (Bax^{+/+}) or Bax-deficient (Bax^{-/-}) mice. PTPC was extracted from the brains of Bax^{+/+} or Bax^{-/-} mice, reconstituted into liposomes, and tested for a response to Atr (100 μ M), t-BHP (200 μ M), or diamide (100 μ M), followed by determination of DiOC₆(3) retention as in Fig. 2D. Bax protein or two inactive mutant Bax proteins (Δ IGDE or Δ 5/6) were added to PTPC from Bax^{-/-} animals to restore the Atr response. (B) Failure of mitochondria from Bax^{-/-} mice to respond to Atr. Isolated hepatocyte mitochondria from Bax^{+/+} or Bax^{-/-} animals were treated with Atr (5 mM) or the protonophore CCCP (50 μ M). The $\Delta\psi_m$ was measured with the potential-sensitive fluorochrome chloromethyl-X-rosamine (CMXRos) (100 nM, 15 min, room temperature) and analysis in a FACS Vantage cytofluorometer (Becton Dickinson, $\sim 5 \times 10^4$ mitochondria per curve). Similar results were obtained when, instead of CMXRos, rhodamine 123 quenching (CCCP = 100 %, control = 0%) was measured as in Fig. 2B (92 \pm 4% and 7 \pm 3% for Atr-treated Bax^{+/+} and Bax^{-/-} mitochondria, respectively, mean \pm SEM of triplicates). (C) Failure of Bax to kill ANT-deficient yeast cells. Wild-type yeast cells or cells lacking all three ANT genes were transfected with a galactose-inducible vector pJG-4-5-Bax (or pJG-4-5-vRas as a control, Co.). Bax or vRas expression was induced by switching culture conditions from a glucose-containing to a galactose-containing medium for the indicated period, and the clonogenic potential of yeast cells was assessed (20). Results are expressed as mean \pm SEM of triplicates (SEM bars mostly obscured by symbols) and were reproduced twice.

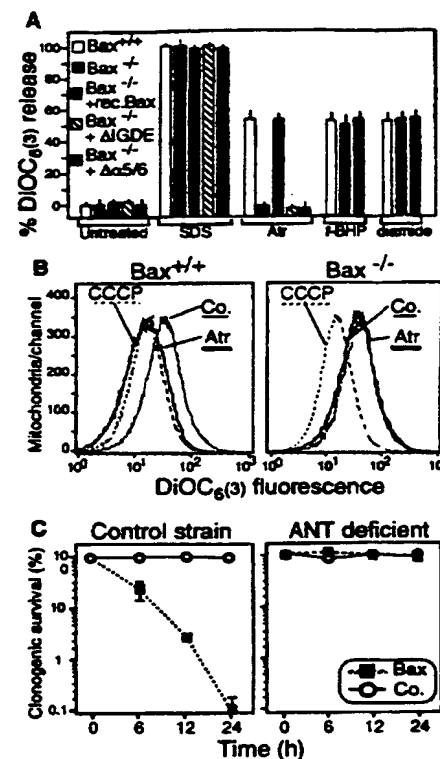
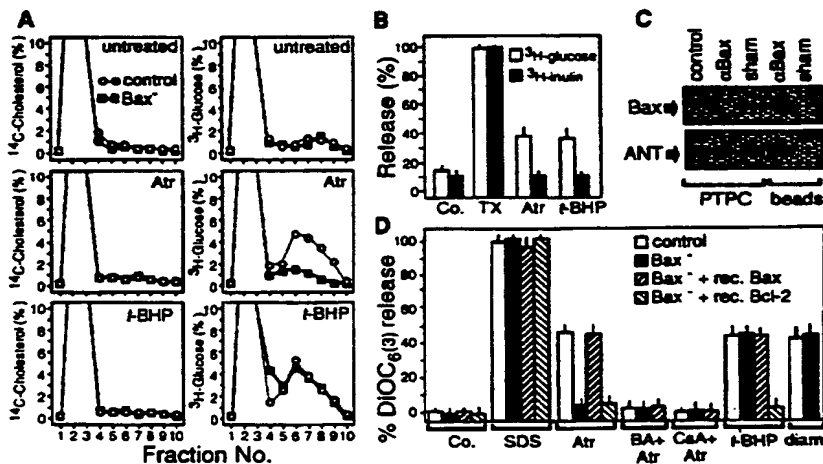


Fig. 2. Bax immunodepletion from PTPC abolishes the Atr-induced permeabilization of liposomal membranes. PTPC liposomes were immunodepleted from Bax, and their functional behavior was compared with control liposomes, which did contain Bax. (A) Bax depletion abolishes the Atr-induced release of [³H]glucose. PTPC immunodepleted of Bax (black squares) or sham-immunodepleted (open circles) was incorporated into liposomes generated in the presence of [¹⁴C]cholesterol (which incorporates into membranes) and [³H]glucose (which marks the aqueous lumen), followed by addition of Atr (500 μ M) or t-BHP (200 μ M), molecular sieve chromatography, and determination of the radioactivity in liposomes (fractions 1 to 4) and the extraliposomal fluid (fractions 6 to 10). (B) Selectivity of the PTPC. Either [³H]glucose or [³H]inulin was encapsulated into control proteoliposomes. Then, the release of either of the radioactive substances [sum of fractions 6 to 10 as in (A)] was determined as in (A) in response to the detergent Triton X-100 (TX, final concentration 2%), Atr (500 μ M), or t-BHP (200 μ M). (C) Immunodepletion of Bax from PTPC. PTPC was enriched by anion exchange chromatography, followed by immunodepletion of Bax or sham-immunodepletion with protein A and G agarose beads. The proteins remaining in PTPC or those attached to agarose beads were subjected to immunodetection of Bax or ANT. (D) Bax depletion selectively blocks the Atr-triggered release of DiOC₆(3) from PTPC proteoliposomes. Bax-immunodepleted (Bax⁻) or control PTPC liposomes were treated with Atr [100 μ M; as in (D)], t-BHP (200 μ M), diamide (diam.) (100 μ M), CsA (1 μ M), or BA (50 μ M), equilibrated with DiOC₆(3), and examined for DiOC₆(3) retention in the cytofluorometer (rec., recombinant). Triplicates of 5×10^4 liposomes were analyzed and results were expressed as percent of reduction of DiOC₆(3) fluorescence, considering the reduction obtained with 0.25% SDS in PTPC liposomes as 100% value, as described (18). Results are representative of three to five independent determinations.



Atr facilitated the ANT-Bax-dependent increase in membrane permeability (Figs. 2 to 4), whereas BA had the opposite effect (Figs. 1 and 2D). Atr binds to the external adenosine diphosphate (ADP)-binding site of ANT and locks it in the "c-state," which is compatible with PT pore opening. In contrast, BA binds to the internal adenosine triphosphate (ATP)-binding site of ANT and locks it in the "m-state," which prevents PT pore opening in response to a wide array of agents (2, 12, 13). Thus, the ANT

conformation evidently determines pore formation by the Bax-ANT complex. The endogenous modulators of ANT conformation may include ATP/ADP ratios, Ca^{2+} , cellular redox status, and fatty acid metabolites (23, 25, 27, 28). By analogy to some receptor complexes, the PTPC could be organized into several functional units, which are responsible for distinct biological responses. In this scenario, interacting pairs of ANT-Bax molecules would form one particular pore-forming unit. Additional units regulated by other physiological effectors could arise from interactions of ANT (or ANT-like mitochondrial solute carriers) with outer membrane proteins, including porin (14, 28) or perhaps proteins of the mitochondrial protein import machinery (29). Several ANT-like proteins can form large conductance pores (30) and are targets of diamide (11, 31), a PT pore-opening agent not influenced by Bax (Figs. 2D and 3A) or Bcl-2 (14, 15). Hence, it is possible that different combinations of inner and outer membrane proteins simultaneously regulate mitochondrial membrane permeability and respond to distinct proapoptotic signal transduc-

Given that both ANT and Bax can form pores (23–25), it is conceivable that the ANT-Bax complex could form a gap junction-like conduit at the inner-outer membrane contact site or regulate the permeability of the outer and inner mitochondrial membranes in a coordinate fashion, or both. Alternatively, under some circumstances, Bax could translocate to and act primarily on the inner membrane in a colicin-like fashion. This latter possibility is underscored by immunoelectron microscopy data indicating that, upon treatment with Atr, Bax redistributes from the outer to the inner mitochondrial membrane. This redistribution has a diffuse rather than clustered pattern and is inhibited by CsA (Fig. 4D). Moreover, when added to mitochondria, Bax and Bcl-2, but not the mutant proteins Bax $\Delta\alpha 5/6$ or Bcl-2 $\Delta\alpha 5/6$, interacted with the mitochondrial inner membrane in a CsA-inhibitable fashion (Fig. 4E), suggesting that the interaction of Bax with the inner membrane depends on the conformation of the PTPC. A subtle derangement in volume homeostasis of the mitochondrial matrix facilitated by Bax could provoke the local mechanical disruption of the outer membrane, as this has been suggested by the study of mitochondrial ultrastructure in apoptotic cells (5). This may explain why opening of the PT pore, which does not allow for the transit of molecules like inulin (5200 daltons; Fig. 2B) or cytochrome c (14 kD) (14), can trigger the release of intermembrane proteins through the outer membrane. Alternatively, or in addition, Bax may undergo ANT-dependent conformational changes or oligomerization events that permit it to form protein-translocating channels in the outer membrane (8).

In conclusion, PTPC may contain several channel-forming proteins that cooperate to mediate apoptosis responses. One such pair of cooperating proteins comprises the constitutive mitochondrial inner membrane protein ANT and the proapoptotic Bcl-2 analog Bax. This discovery provides a biochemical and mechanistic link between the PT pore and the mode of action of apoptosis regulatory proteins of the Bcl-2-Bax family.

A

α Bax α Bcl-2 α ANT α all-4

B

LexA	β -Gal with TA constructs	
	+	-
Bax	ANT, Bcl-2	Ras
Bak	ANT, Bcl-2	Ras
Bcl-2	ANT, Bcl-2	Ras
ANT	ANT, Bax, Bak, Bcl-2	Ras
Fas, Traf-3		ANT, Bax, Bak

C

Atr (μM)	Bax + ANT	ANT	Δ IGDE + ANT	$\Delta\alpha 5/6$ + ANT	Bcl-2 + ANT	Bax
0	0	0	0	0	0	0
6	15	5	5	5	2	2
12	35	8	8	8	4	4
25	45	10	10	10	6	6
50	48	12	12	12	8	8
100	49	14	14	14	10	10

D

Condition	ext.	int.
Co.	1.5	1.3
Atr.	0.4	2.8
CsA + Atr	1.4	1.4

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Z

AA

AB

AC

AD

AE

AF

AG

AH

AI

AJ

AK

AL

AM

AN

AO

AP

AQ

AR

AS

AT

AU

AV

AW

AX

results, are listed. (C) Bax and ANT cooperate to permeabilize membranes in response to Atr. Recombinant purified Bax, two inactive mutant Bax proteins (Δ IGDE or $\Delta\alpha 5/6$), Bcl-2, and purified ANT from rat myocardium (24) were incorporated into phosphatidylcholine-cardiolipin (1:46 w/w) vesicles (300 ng of ANT per milligram of lipid; 50 ng of Bax, Bax Δ IGDE, Bax $\Delta\alpha 5/6$, or Bcl-2 per milligram of lipid) either alone or together. The response to different doses of Atr was evaluated by measuring the DiOC6(3) retention as in Fig. 2D. Results are representative of three independent experiments. (D) Submitochondrial redistribution of Bax upon treatment with Atr. Purified mouse liver mitochondria were incubated during 30 min with 5 mM Atr or 1 μ M CsA or both, fixed, and subjected to immunoelectron microscopy for the detection of Bax [α -Bax plus Immunogold (5 nm) anti-rabbit immunoglobulin conjugate]. Representative micrographs counterstained with uranium acetate are shown. The number of grains located on the internal (hatched bars) or external (white bars) mitochondrial membrane were counted. Numbers indicate the number of grains per mitochondrion (mean \pm SEM, $n = 100$; background value obtained with preimmune serum <0.1). (E) Interaction of Bax and Bcl-2 protein with the inner mitochondrial membrane. Mouse liver mitoplasts (35) (50 μ g of protein in 50 μ l; same buffer as in Fig. 1B) were incubated in the absence (Co.) or presence of 1 μ M Bax, Bax $\Delta\alpha 5/6$, Bcl-2, or Bcl-2 $\Delta\alpha 5/6$ protein (30 min, 37°C), in the presence or absence of 1 μ M CsA (denoted by + or -), washed (10 min, 10,000g, 4°C) three times, subjected to SDS-PAGE and immunodetection of Bax or Bcl-2 with antibodies recognizing both the native and the $\Delta\alpha 5/6$ proteins.

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10. Recombinant human Bcl-2(1-218) or mouse Bax(1-171) proteins lacking the hydrophobic transmembrane (TM) domain [deletion of amino acids 219 through 239 (Δ 219-239) in the case of Bcl-2; Δ 172-192 for Bax], Bcl-2 Δ 5/6[Bcl-2(Δ 143-184)], Bax Δ IGDE (Δ 66-69), and Bax Δ 5/6 (Δ 106-153) were created with a two-step polymerase chain reaction (PCR) method [H. Zha et al., *Mol. Cell. Biol.* 16, 6494 (1996)]. These five proteins were then tagged with His₆ or glutathione S-transferase (in the case of Bax Δ 5/6) and purified as described [(8); S. Schendel et al., *Proc. Natl. Acad. Sci. U.S.A.* 94, 5113 (1997); Z. H. Xie, S. Schendel, S. Matsuyama, J. C. Reed, *Biochemistry* 37, 6410 (1998)].
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16. PTPC was prepared from Wistar rat brains or from Bax^{-/-} [C. M. Knudson, K. S. Tung, W. G. Tourtellotte, G. A. Brown, S. J. Korsmeyer, *Science* 270, 96 (1995)] or Bax^{+/+} C57BL/6 mouse brains (14). For Bax immunodepletion the PTPC preparation was incubated with polyclonal antibody to Bax (anti-Bax; 50 μ g/ml) (Δ 21, Santa-Cruz) for 2 hours at room temperature. Sham immunodepletion was done with a preimmune rabbit antiserum. A 0.1 volume of protein A and protein G agarose beads was added for 30 min and removed by centrifugation (10 min, 2000g). PTPC in the supernatant (~1 μ g of protein per milligram of lipid) was reconstituted into lipid vesicles (optionally supplemented with 0.5 μ Ci of [¹⁴C]cholesterol per 100 mg of lipids) by overnight dialysis. Recombinant proteins were added during the dialysis step at a dose corresponding to 1% (Bax or Bax mutants) or 5% (Bcl-2) of total PTPC proteins. Liposomes were ultrasonicated in 5 mM malate and 10 mM KCl (optionally supplemented with 5 μ Ci of [³H]glucose per milliliter or 5 μ Ci of [³H]inulin per milliliter) and separated on Sephadex G50 columns (14). Proteoliposomes containing maximum hexokinase activity (14) were incubated (60 min, room temperature) with PT pore-opening agents in 125 mM sucrose plus 10 mM Hepes (pH 7.4). When the release of [³H]glucose or [³H]inulin was assessed, liposomes were separated on Sephadex G50 Nick columns (Pharmacia). Alternatively, liposomes were equilibrated for 30 min with 80 mM 3,3'-diethylocarbocyanine [DiOC₂(3)] and then analyzed in a cytofluorometer (14).
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20. The *S. cerevisiae* W301-1B control strain (MATa, ade2, trp1, leu2, ura3, his3) and the triple ANT mutant JL-1-3 strain (MATa, LEU::aac1, HIS::aac2, URA::aac3, ade2, trp1, leu2, ura3, his3) [T. Orgon, L. Sabova, N. Nelson, J. Kolarov, *FEBS Lett.* 289, 159 (1991)] were transfected with a pJG-4-5 vector containing the Trp1 selection marker and murine Bax under the control of the Gal promoter, and the lethal effect of Bax overexpression (controlled by immunoblot) was assessed [S. Matsuyama, Q. Xu, J. Velours, J. C. Reed, *Mol. Cell* 1, 327 (1998)]. The pJG-4-5 vector containing v-Ras instead of Bax served as a negative control.
21. Cells or organs were homogenized in H buffer (150 μ M MgCl₂, 10 mM KCl, 10 mM tris-HCl pH 7.6), followed by elimination of nuclei (3 min at 900g, at 4°C, recovery of supernatant), elimination of soluble proteins (10 min at 6800g), resuspension of the mitochondria-containing pellet in H buffer supplemented with 0.5% Triton (v/v, final concentration; 0.5 mg of protein per milliliter), addition of polyclonal rabbit antiserum (1 μ g of antibody per 10 μ g of protein in sample) specific for Bcl-2, Bax (Santa Cruz Biotechnology), human interleukin-4 (IL-4) (Pharmingen), or ANT [J. Gironcallo and H. H. O. Schmid, *Biochemistry* 35, 15440 (1996)], incubation for 90 min at 37°C, addition of 10% protein A and G agarose beads (Santa Cruz Biotechnology) for 30 min at 37°C, recovery (10 min, 2000g) of the washed (two times in phosphate-buffered saline, pH 7.4) beads in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and immunodetection with monoclonal antibody (mAb) to Bax (P19, Santa Cruz), mAb to Bcl-2 (clone 124, Dako), or antiserum to ANT.
22. A human Bax cDNA without TM [J. M. Jürgensmeier et al., *Mol. Biol. Cell* 8, 325 (1997)], human Bcl-2 lacking the TM, murine Bax cDNA lacking the TM, Ras, Fas, Traf-3, or a human ANT2 cDNA fragment (amino acids 105 to 156) were subcloned in the Eco R1-Xho I sites of pGilda or pJG4-5. EGY48 cells were used for lacZ reporter gene assays in conjunction with pGilda (LexA DNA-binding domain) and pJG4-5 (B42 transactivation domain) and psh 18-34 reporter plasmids. Filter assays were performed for galactosidase measurement, with cells plated on either galactose- or glucose-containing minimal medium (19) [M. Hanada, C. Aime-Sempé, T. Sato, J. C. Reed, *J. Biol. Chem.* 270, 11962 (1995); H. Zha, C. Aime-Sempé, T. Sato, J. C. Reed, *ibid.* 271, 7440 (1996)].
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36. We thank D. Andrews for Rat-1 cells; D. Brdiczka for help in ANT purification; T. Orgon for ANT-deficient yeast; H. J. Duine for BA; S. Korsmeyer for Bax^{-/-} mice; N. Roy for ANT plasmid; H. Schmid for the ANT-specific antibody; A. Zweibaum for HT29 cells; and N. Larochette and D. Mátivier for technical assistance. Supported by Agence Nationale pour la Recherche sur le SIDA, Association pour la Recherche sur le Cancer, CNRS, Fondation pour la Recherche Médicale, and Ligue Française contre le Cancer (to G.J.), and University of California Breast Cancer Research Program (grant number IR8-0098) and CapCure Incorporated (to J.C.R.). S.A.S. and E.L.A.V. receive fellowships from the European Commission, I.M. from the Spanish Ministry of Science, and J.M.J. from the Deutsche Forschungsgemeinschaft.

21 May 1998; accepted 12 August 1998

Dorsal-Ventral Signaling in the *Drosophila* Eye

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The development of the *Drosophila* eye has served as a model system for investigations of tissue patterning and cell-cell communication; however, early eye development has not been well understood. The results presented here indicate that specialized cells are established along the dorsal-ventral midline of the developing eye by Notch-mediated signaling between dorsal and ventral cells, and that Notch activation at the midline plays an essential role both in promoting the growth of the eye primordia and in regulating eye patterning. These observations imply that the developmental homology between *Drosophila* wings and vertebrate limbs extends to *Drosophila* eyes.

Patterning and growth of the *Drosophila* wing depend on signaling between cells in different compartments (1, 2). This signaling establishes specialized cells along compartment boundaries, which in turn secrete mol-

ecules that regulate wing development. The *fringe* (*fng*) gene regulates signaling between dorsal and ventral cells (3). Fringe protein (FNG) is produced by dorsal cells and inhibits cells' responsiveness to the Notch ligand Serrate (SER), while potentiating cells' responsiveness to the Notch ligand Delta (DL) (4, 5). This results in activation of Notch along the *fng* expression border, which normally corresponds to the dorsal-ventral (D-V) compartment boundary. Local activation of Notch in the wing is both necessary and sufficient for long-range effects on the fate and proliferation of wing cells (1, 2).

Strict compartment boundaries have not

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